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Genes Expressed in the Amphioxus Notochord Revealed by EST Analysis

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The notochord cell of the cephalochordate amphioxus adult is unique due to the occurrence of myofilaments in the cytoplasm. The present EST (expressed sequence tag) analysis targeted mRNAs of the amphioxus notochord to determine genes that are expressed there. Notochord cells were isolated from *Branchiostoma belcheri* adults, from which a cDNA library was constructed. Analysis of a set of 257 ESTs (both 5′ and 3′ ends) showed that about 11% of the cDNAs are related to muscle genes, while 9% of them are genes for extracellular matrix proteins associated with formation of the notochordal sheath. The muscle-related genes included actin, tropomyosin, troponin I, myosin regulatory light chain, myosin light chain kinase, myosin heavy chain, calmodulin, calponin, calcium vector protein, creatine kinase, muscle LIM protein, and SH3-binding glutamate-rich protein, suggesting that vertebrate skeletal and smooth muscle-type genes are simultaneously expressed in the amphioxus notochord. Nucleotide sequences of cDNAs for actin, tropomyosin, troponin I, and a few others were completely determined to substantiate the conclusions. The chordate muscle-type actin is distinguishable from the cytoplasmic-type actin by the usage of amino acid residues at 20 diagnostic positions. Interestingly, analysis of the usage of amino acid residues at these positions showed that the "amphioxus notochord actin" is a unique intermediate between muscle-type and cytoplasmic-type actins. These results strongly suggest that the notochord of adult amphioxus is a mechanical swimming organ and its role is quite different from the role of the vertebrate embryonic notochord, which functions as a source of signals required for body plan formation.

Key Words: **amphioxus; notochord; EST analysis; muscle genes; actins; notochordal sheath; extracellular matrix; chordate evolution.**

INTRODUCTION

The evolution of chordate body plans has been debated for more than a century (Gee, 1996; Nielsen, 1999). The notochord is a defining characteristic of chordates (Satoh and Jeffery, 1995; Satoh *et al.*, 1999). It is a dorsally located rod of tensile mesodermal tissue that lies immediately beneath the neural tube. The notochord functions as a skeletal element during early chordate embryogenesis. In addition, in vertebrate embryos, the notochord functions as a source of signals that determine the patterns of the neural tube and paraxial mesoderm. Despite the recent molecular identification of mutations affecting notochord development in mice (Herrmann *et al.*, 1990) and in zebrafish (Odenthal *et al.*, 1996; Stemple *et al.*, 1996), the rather modest progress in isolating genes expressed in the notochord during its differentiation has limited our understanding of the molecular mechanisms underlying its structure and function, as was pointed out by Cunliffe and Ingham (1999).

The cephalochordate amphioxus belongs to an invertebrate group closest to vertebrates, as was suggested by recent molecular phylogenetic analyses (Wada and Satoh, 1994; Turbeville *et al.*, 1994). The amphioxus notochord is a solid rod of tissue that is situated in the middle of the body between the dorsal wall of the gut and the ventral wall of the nerve cord. It is enclosed in a well-developed extracellular sheath (cf. Fig. 1A). However, the amphioxus notochord is unique with respect to at least three points (Ruppert, 1997). First, unlike the notochords of all other chordates, the cephalochordate notochord extends from the tip of the tail to the tip of the rostrum. This extreme anterior extent of the notochord is a hallmark of amphioxus

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and is responsible for the name "Cephalochordata." Second, the amphioxus notochord develops as an outfold of the roof of the archenteron that separates from the embryonic gut as a distinct structure (Conklin, 1932; Hirakow and Kajita, 1994). Each notochord cell produces a large central vacuole, which displaces the nucleus to the periphery of the cell, and then the cells become discoidal in shape as they arrange themselves single file in a longitudinal series. Third and most prominent, the amphioxus notochord cells are a type of muscle cell. As early as 1871, Müller (1871) described, as structural components of the amphioxus notochord, the lamellae which contain horizontally arranged birefringent filaments. Later studies using electron microscopy, cytochemistry, electrophysiology, and cinephotography revealed that the notochord cells of amphioxus contain myofilaments and that the organ is capable of altering its mechanical properties upon nervous stimulation (Flood *et al.*, 1969; Flood, 1975).

However, very few molecular, developmental biological studies have attempted to demonstrate the expression of genes in the amphioxus notochord, although *Brachyury* is expressed in the developing notochord (P. Holland *et al.*, 1995; Terazawa and Satoh, 1997). To address this question, the present study adopted a method of expressed sequence tag (EST) analysis and was able to reveal that at least 12 different types of muscle-related genes are expressed in the amphioxus notochord.

MATERIALS AND METHODS

Biological materials. Adults of the amphioxus *Branchiostoma belcheri* were collected near the National Research Institute of Aquaculture, Mie, Japan and also provided by Dr. Kaoru Kubokawa of the Ocean Research Institute of the University of Tokyo. The notochord region of adults was roughly dissected (Fig. 1A). Then cells of other tissues around the notochordal sheath were completely removed. Subsequently, only notochord cells were squeezed out of the sheath with tweezers (Fig. 1B) and collected with a micropipette. Notochord cells were collected from about 200 adults.

Library construction. Total RNA was isolated from notochord cells by the acid guanidinium thiocyanate–phenol–chloroform method. Poly(A)$^+$ RNA was purified using Oligotex beads (Roche Japan, Tokyo). Poly(A)$^+$ RNA was converted to double-stranded cDNA which contained an *Eco*RI site at the 5′ end and an *Xho*I site at the 3′ end and ligated to Uni-ZAP XR vector using a ZAP cDNA synthesis kit (Stratagene, La Jolla, CA). The resulting library contained 1.6×10^6 clones. cDNA libraries of amphioxus muscle and ovary were constructed by the same method as described above. They contained 6.4×10^5 and 1.8×10^5 clones, respectively.

Sequence determination and search. pBluescript SK(−) phagemid sequences within Lambda ZAP vectors were excised using a rapid excision kit (Stratagene). The 5′ and 3′ ends of each cDNA were sequenced using a Big-Dye Terminator Cycle Sequencing Ready Reaction kit and ABI PRISM 377 DNA sequencer (Perkin–Elmer, Norwalk, CT). Insert lengths in this library were mainly in the range 1.2–2.5 kb, and the average readable EST length on which the following analysis is based was about 600 nucleotides. Each of the nucleotide sequences was used as a query sequence for BLASTX programs against peptide sequence databases (nonredundant GenBank CDS translations, PDB, SwissProt, PIR, and PRF).

In addition, cDNA clones for six muscle-related genes were completely sequenced from both strands.

Whole-mount in situ hybridization. To determine mRNA distribution, RNA probes were prepared with a DIG RNA Labeling Kit (Boehringer Mannheim, Heidelberg, Germany). Whole-mount *in situ* hybridization was performed using slices of the amphioxus adult body and digoxigenin-labeled antisense probes as described by Holland *et al.* (1996), with some modification in proteinase K treatment, prehybridization, and hybridization steps according to Bueno *et al.* (1997). Control slices hybridized with sense probes did not show signals above background.

Polymerase chain reaction (PCR). The cDNA libraries of notochord, muscle, and ovary were amplified on plates to 10^9 pfu/ml. Half a microliter of each of the amplified libraries was used as template for the subsequent PCRs. PCRs were performed in a total volume of 10 μl and the conditions were 35 cycles of 94°C for 1 min, 54°C for 2 min, 72°C for 1 min. The specific primers used were as follows: adult muscle actin (F, 5′-TAGCCTGTTCTGGTTGC-3′; R, 5′-CAGACAGAATAGCGTCCG-3′), 01C08 actin (F, 5′-ATTGTCCACCGCAAGTGC-3′; R, 5′-CACCAACAGCGTCAGGC-3′), 01F12 actin (F, 5′-ACAAACGCAACGCAACCC-3′; R, 5′-GACATAACTTAAGGGATG-3′), troponin I (F, 5′-TGTCCCGGTAATAGTTCAG-3′; R, 5′-TATGTTGGCTATTCTACG-3′), calmodulin (F, 5′-CTTTGTTGCATGGTGGTC-3′; R, 5′-CCCGGTACTCCAATGTAC-3′), myosin regulatory light chain (F, 5′-TGCCGCGTAAGAAGACAG-3′; R, 5′-TGGATCCGTACCTCCCAG-3′), CAVP (F, 5′-AGATCCTACAAGTAACTC-3′; R, 5′-CCGGTCACCAACCTAGTC-3′), creatine kinase (F, 5′-TTGTCCAAGCACAACAA-3′; R, 5′-TCTCGTCGAAGTCACAAC-3′), and muscle LIM protein (F, 5′-TTGCAACTGTGAGGTAC-3′; R, 5′-TATGTCACTCACGATGCG-3′).

RESULTS

EST Analysis of Genes Expressed in the Amphioxus Notochord

Nucleotide sequences of about 600 bp of both the 5′ and the 3′ ends of 257 cDNA clones that were randomly selected from a *B. belcheri* notochord library were determined. The translated sequences were compared to GenBank and significant matches collected. Expected values (*E*) lower than 10^{-6} were categorized as those of "sequence similarity," while those higher than 10^{-5} were categorized as "no sequence similarity." Hitherto in the amphioxus, cDNA clones for only four muscle-related genes were characterized, namely muscle actin (R. Kusakabe *et al.*, 1997, 1999), alkali myosin light chain (L. Holland *et al.*, 1995), troponin C (Yuasa *et al.*, 1998), and calcium vector proteins (CAVP) (Yuasa *et al.*, 1999).

As shown in Fig. 2, database searches indicated that the deduced amino acid sequences of half (128) of the cDNAs had similarity to those of reported proteins, while the other half (129 cDNAs) showed no similarity using the present sequence determination and database analysis. cDNAs with sequence similarity were categorized into genes related to muscle (27 clones, 11%), genes for extracellular matrix protein (16 clones, 6%), genes for ribosomal proteins

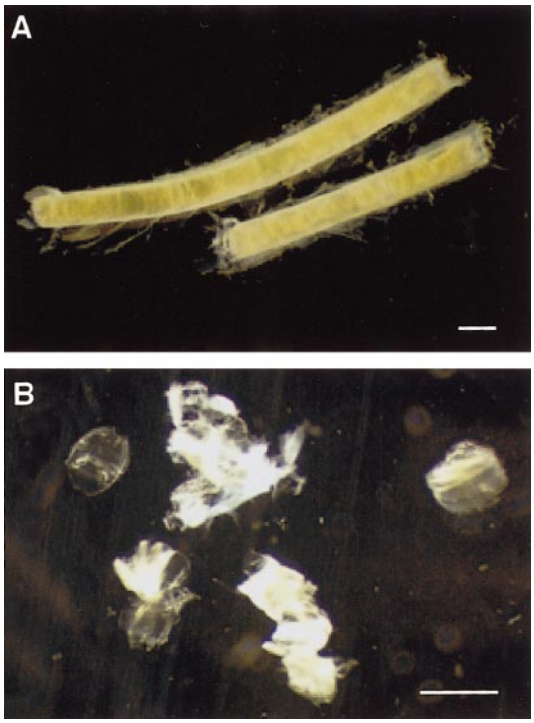


FIG. 1. Isolation of notochord cells from amphioxus adults. (A) Notochord enclosed within the sheath was isolated by dissection of the adult body. (B) Isolated notochord cells used for construction of cDNA library. Scale bars, 1 mm.

(14 clones, 5%), genes for the immune system (7 clones, 3%), and genes for other proteins (64 clones, 25%). These results, together with the determination of the complete nucleotide sequences of several cDNAs described below, clearly indicate that various kinds of muscle-related genes are expressed in the amphioxus notochord. Details of analysis of cDNA clones related to the immune system will be described elsewhere (Suzuki *et al.*, unpublished). The category "others" includes genes for ferritin, bFGF, agrin, ceruloplasmin, ubiquitin, HSP70, and ATP sulfurylase/APS kinase.

Expression of Muscle-Related Genes

The present EST analysis showed that 27 cDNA clones represented 12 genes that are associated with the formation and function of muscle cells (Table 1).

Actin genes. Seven cDNAs (01C08, 01F12, 02D09, 02A11, 03H03, 03A06, and 03C03) encoded actin. Determination of the complete nucleotide sequences of the first five cDNAs demonstrated that all of them encoded an identical actin composed of 376 amino acid residues. In addition, 02D09, 02A11, and 03H03 were almost identical in their 5′ and 3′ UTR nucleotide sequences (Fig. 3A). Small differences among the three clones may have been caused by polymorphism, because notochord cells for mRNA isolation were collected from about 200 individuals (Materials and Methods). However, 01C08, 01F12, and 02D09 differed from each other in their 5′ and 3′ UTR sequences (Fig. 3A). It is likely that these three transcripts are transcribed from different actin genes. As shown in Fig. 4C, *in situ* hybridization showed that the occurrence of transcripts of the 01F12 actin gene was specific to the notochord cells. The 01C08, 02D09, 02A11, and 03H03 genes showed the same result (data not shown). We performed PCR analyses of cDNA libraries of notochord, muscle, and ovary with specific primers to the 3′ UTR sequences. The results indicated that 01F12 actin is expressed exclusively in the notochord (Fig. 4C). However, PCR analysis indicated that 01C08 and other actin genes are expressed in the muscle as well (data not shown).

In vertebrates, the α-skeletal muscle actin is distinguishable from the β-cytoplasmic actin by the usage of amino acid residues at 20 diagnostic positions (Vandekerckhove and Weber, 1979, 1984). Comparison of the amino acid residues of the amphioxus notochord actin showed that the amphioxus notochord actin shared 10 of the 20 diagnostic amino acids with the mammalian β-cytoplasmic actin and 9 of the 20 diagnostic amino acids with the mammalian α-skeletal actin (Fig. 3B and dots in Fig. 3C). This suggests that the actin expressed in the amphioxus notochord is neither of cytoplasmic type nor of muscle type, but rather that it is a unique one that should be called "the amphioxus notochord actin (BbNA1)."

In addition, as shown in Fig. 3C, there are 68 amino acid positions that are not conserved in amphioxus actins. These amino acid residues are used to deduce relationships between the notochord (BbNA1), the cytoplasmic (BbCA1 and BfCA1), and the muscle (BbMA1 and BfMA1) actins. BbNA1 actin shares 35 of the 68 amino acid residues with BbCA1 and BfCA1 (blue in Fig. 3C) and 9 of the 68 amino acids with

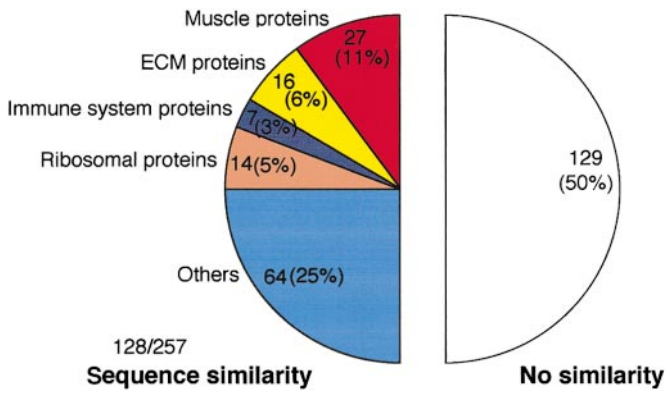
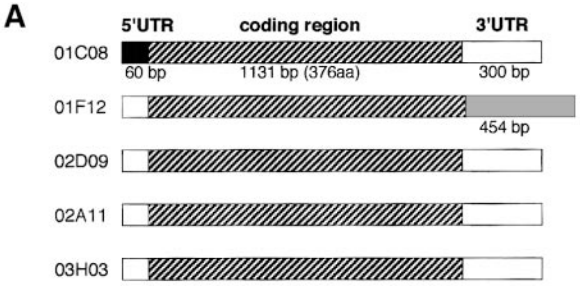
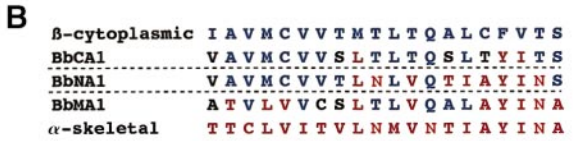
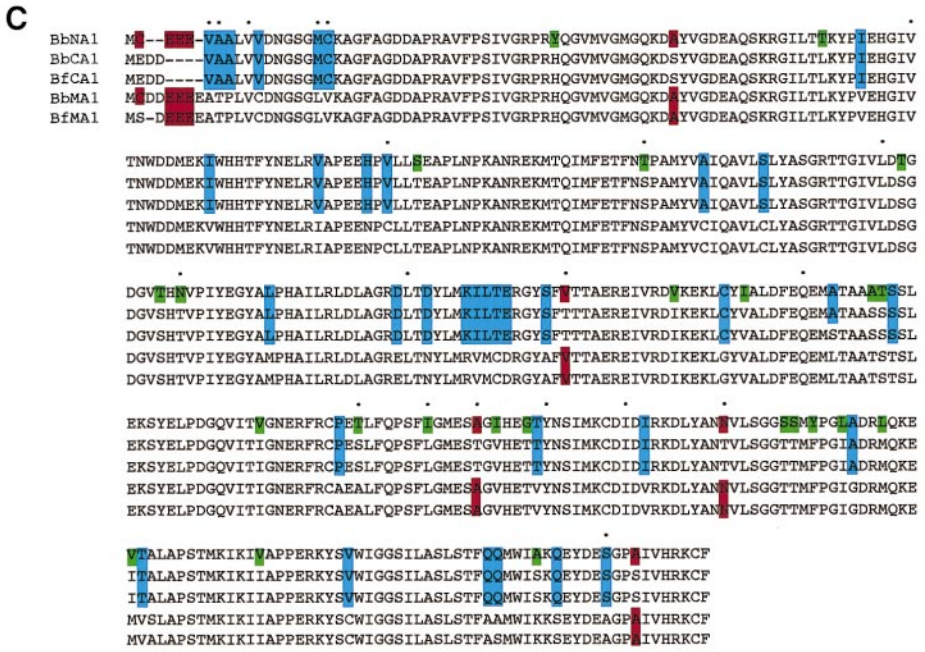


FIG. 2. Summary of EST analysis of 257 cDNA clones obtained from amphioxus notochord. Of them, 128 clones showed amino acid sequence similarity to reported proteins, while the other 129 clones had no such sequence similarity.







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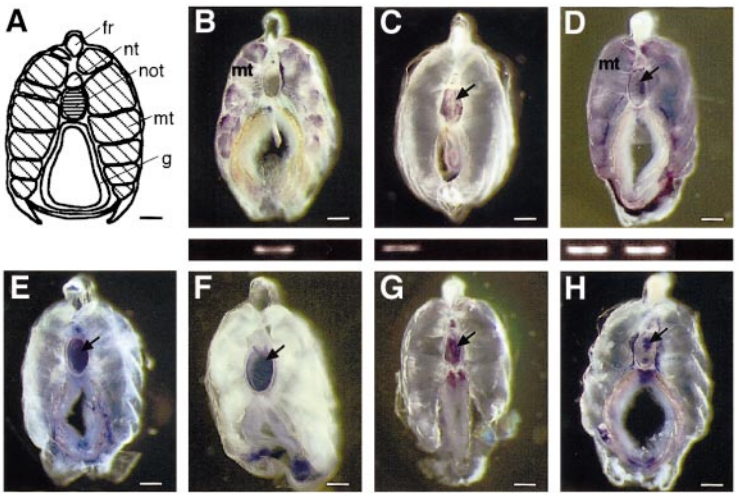

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TABLE 1
cDNA Clones for Muscle-Related Genes Expressed in the Amphioxus Notochord

	No. of clones	Accession No.
I. Actin		
Notochord actin (BbNA1)[a]	7	AB035660, AB035661, and AB035662
II. Muscle contraction regulatory proteins		
Tropomyosin[a]	2	AB035663
Troponin I[a]	2	AB035664
Calmodulin	4	
Calponin	2	
Myosin light chain kinase	1	
Myosin regulatory light chain[a]	1	AB035665
Myosin heavy chain	2	
IV. Others		
CAVP[a]	2	AB035666
Creatine kinase	2	
Muscle LIM protein[a]	1	AB035667
SH3BGR	1	

[a] cDNA clones encoding full ORF and completely sequenced.

BbMA1 and BfMA1 (red in Fig. 3C). However, BbNA1 actin contains 24 of the 68 amino acid residues different from those of the cytoplasmic and muscle actins (green in Fig. 3C).

Genes for muscle proteins associated with regulation of contraction. In addition to the actins described above, the present EST analysis suggested that 14 clones represented genes associated with muscle contraction. As summarized in Table 1, they included tropomyosin (2 clones), troponin I (2 clones), calmodulin (4 clones), calponin (2 clones), myosin light chain kinase (1 clone), myosin regulatory light chain (1 clone), and myosin heavy chain (2 clones). Of them, three cDNAs were completely sequenced, and the sequences revealed that tropomyosin (Fig. 5A), troponin I (Fig. 5B), and myosin regulatory light chains (Fig. 5C) are authentic amphioxus homologs of the vertebrate muscle genes.

Whole-mount *in situ* hybridization showed that all of the genes were expressed in the notochord; troponin I in Fig. 4D, calmodulin in Fig. 4E, and myosin regulatory light chain in Fig. 4F. Although hybridization signals appeared to be specific to the notochord, PCR analysis suggested that transcripts of these genes were present in the myotome (Fig. 4D for troponin I). *In situ* hybridization, however, suggested that the level of mRNA in the notochord was higher than that in muscle, because the signals were much stronger in the notochord.

Genes for muscle proteins with other functions. The sequences of five cDNA clones suggested that two were genes for CAVP, two for creatine kinase, one for muscle LIM protein, and one for SH3-binding glutamate-rich protein (SH3BGR) (Table 1). CAVP is an EF-hand Ca^{2+}-binding protein for muscle and is thought to act as a Ca^{2+} signal transducer (Kobayashi *et al.*, 1987). The complete nucleotide sequence of the CAVP cDNA was determined. The entire amino acid sequence of CAVP of *B. belcheri* showed 98 and 96% identity with those of *B. floridae* and *B. lanceolatum* (Yuasa *et al.*, 1999) but had an additional five amino acids at the N-terminus (Fig. 5D). Creatine kinase is an enzyme which plays a central role in energy transduction in tissues with large, fluctuating energy demands, such as skeletal muscle, heart, brain, and spermatozoa. Creatine kinase consists of an M chain and a B chain and acts as a dimer, MM being the major form in skeletal muscle and myocardium (Wallimann, 1994). The amphioxus notochord creatine kinase shows the most similarity with vertebrate M chain.

Muscle LIM protein, originally isolated from *Drosophila*, plays a role in cell differentiation later in myogenesis (Arber *et al.*, 1994). The complete nucleotide sequence of the cDNA of the amphioxus muscle LIM protein was deter-

FIG. 3. Characterization of amphioxus notochord actin mRNAs. Nucleotide sequences of all of the five cDNAs were completely determined. (A) Diagrammatic representation of the five cDNAs showing that they encode an identical actin consisting of 376 amino acids. The nucleotide sequences of the 5′ and 3′ UTRs of 02D09, 02A11, and 03H03 were almost identical, while 01C08 and 01F12 had different 5′ and 3′ UTRs, respectively. (B) Comparison of amino acid residues at 20 diagnostic positions according to Vandekerckhove and Weber (1984): blue for mammalian β-cytoplasmic actin and red for mammalian α-skeletal actin. (C) Alignment of amino acid sequences of an amphioxus notochord actin (BbNA1) with those of cytoplasmic-type actin of *B. belcheri* (BbCA1) and *B. floridae* (BfCA1) and muscle-type actin of *B. belcheri* (BbMA1) and *B. floridae* (BfMA1) (R. Kusakabe *et al.*, 1997, 1999). Red indicates amino acid residues conserved between amphioxus BbNA1 and muscle actins, blue those conserved between BbNA1 and cytoplasmic actins, and green those specific to BbNA1. Black dots indicate the positions of the 20 diagnostic amino acids.

FIG. 4. Expression of muscle-related and ECM-related genes in the amphioxus notochord revealed by whole-mount *in situ* hybridization. (A) Diagram of the cross section of amphioxus adult indicating the position of the notochord (not) and myotome (mt). fr, fin ray; g, gut; and nt, neural tube. (B) A control; a muscle-type actin gene is expressed in the myotome but not in the notochord. Scale bar is 400 μm for all. PCR analysis (below) indicates that the gene is expressed in the muscle (middle lane) but not in the notochord (left lane) or the ovary (right lane). (C) Expression of the 01F12 notochord actin gene in the notochord (arrow) but not in the myotome. The specificity of the gene expression in the notochord was confirmed by PCR analysis (below). (D) Expression of troponin I gene in the notochord (arrow) as well as in the myotome. PCR analysis (below). (E) Calmodulin gene. (F) Myosin regulatory light chain gene. (G) Muscle LIM protein gene. (H) Hemagglutinin and amebocyte aggregation factor gene.

mined and revealed that the protein has two LIM domains, each of which is followed by a conserved glycine-rich repeat (Fig. 5E). This is the same structure as cysteine- and glycine-rich proteins, which are vertebrate muscle LIM protein isoforms.

SH3BGR has been characterized in humans, in which it is expressed in heart and skeletal muscle (Scartezzini *et al.*, 1997).

In situ hybridization showed that signals for mRNAs of CAVP (data not shown), creatine kinase (data not shown), and muscle LIM protein (Fig. 4G) were evident in both the notochord and the myotome, whereas signals for SH3BGR were predominant in the notochord (data not shown).

Genes for Extracellular Matrix (ECM) Proteins

The amphioxus notochord is enclosed in a well-developed extracellular sheath (Fig. 1A). Its ECM components are thought to be produced by the notochord cells. Therefore, it is expected that genes for the ECM proteins are expressed in the notochord cells. The present EST analysis demonstrated the presence of mRNAs for six different genes encoding ECM proteins.

Viteronectin (represented by four cDNA clones) and von Willebrand factor (one clone) are major components of the ECM (Ruoslahti and Pierschbacher, 1987). Eight cDNAs represented a gene with strong similarity to hemagglutinin and amebocyte aggregation factor of *Limulus polyphemus,* which is related to mammalian tyrosine-rich acidic matrix protein (dermatopontin) (Fujii *et al.,* 1992). Two types of matrix metalloproteinase (MMP) were also obtained. MT1-MMP specifically digests type-I, -II, and -III collagen (Ohuchi *et al.,* 1997), whereas MT3-MMP specifically digests type-III collagen (Matsumoto *et al.,* 1997). One cDNA encoded metalloproteinase inhibitor 3, which specifically inhibits the activity of MT-MMPs (Will *et al.,* 1996; Butler *et al.,* 1997).

In situ hybridization showed that all of the genes were expressed mainly in the notochord (Fig. 4H for the hemagglutinin and amebocyte aggregation factor gene). The present EST analysis failed to isolate cDNAs for collagens. However, the presence of MT-MMP mRNAs and MMP inhibitor mRNA suggests the probable inclusion of collagen cDNAs in the library.

DISCUSSION

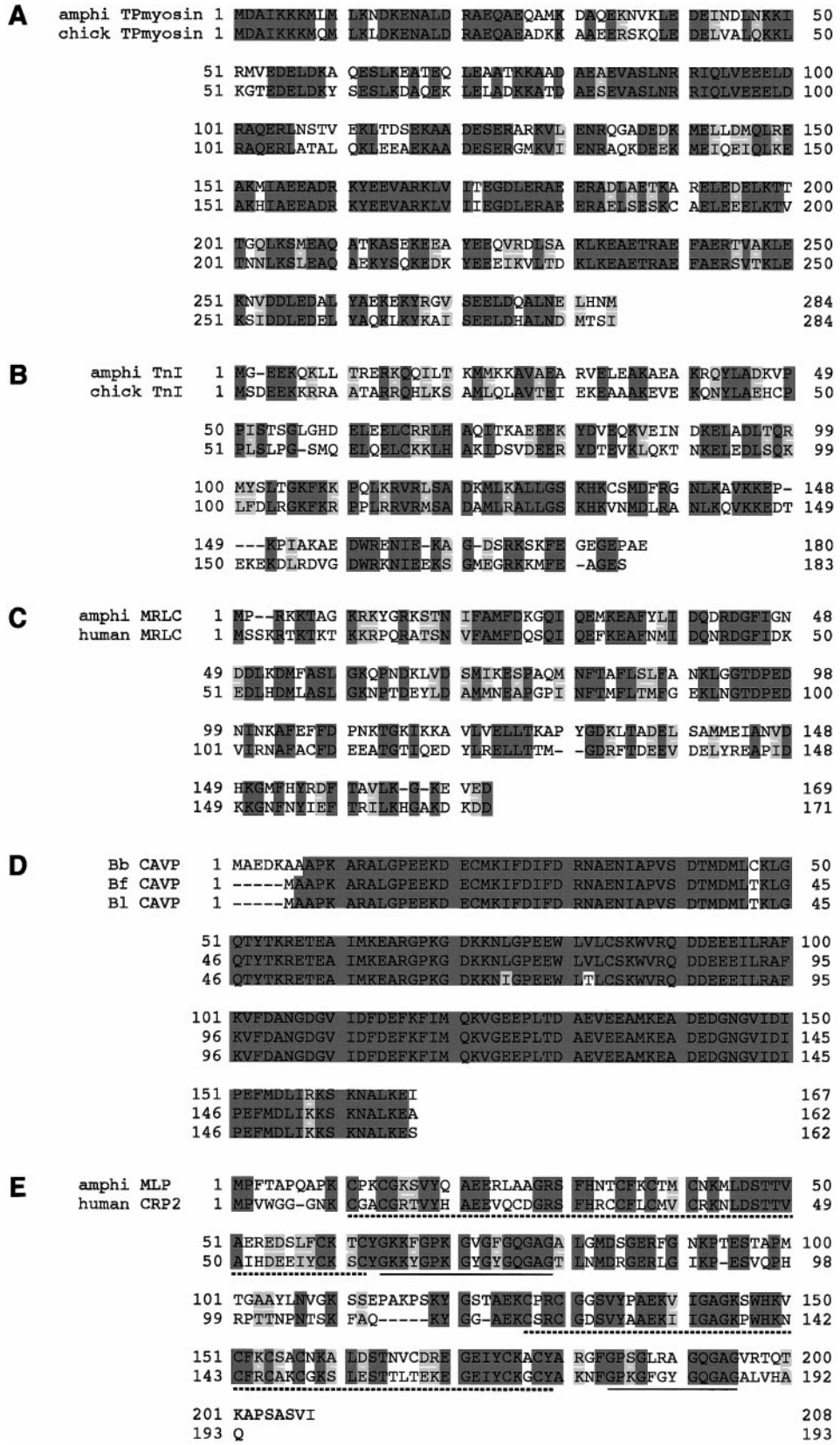
Expression of Muscle-Related Genes in the Amphioxus Notochord

In accordance with the results of previous studies demonstrating the presence of myofilaments in the notochord cells (Flood, 1975), the present EST analysis of genes that are expressed in the amphioxus notochord clearly showed that as many as 11% of mRNAs analyzed encoded muscle-related genes. The cDNA library was made of nonnormalized mRNAs from the amphioxus notochord cells, and we examined cDNAs randomly selected from the library. Therefore, it is obvious that considerable amounts of mRNAs of muscle-related genes are present in the adult amphioxus notochord. Function of the amphioxus notochord cells filled with myofilaments could be maintained by such an active expression of muscle-related genes. When amphioxus notochord is stimulated electrically, the notochord increases its stiffness as a result of muscular contraction (Flood, 1975). As shown in the present study, more than half of the muscle-related genes expressed in amphioxus notochord cells were homologs of vertebrate muscle contraction genes. These results substantiate the contractile properties of the amphioxus notochord.

Muscle contraction is regulated by the concentration of Ca^{2+} within the cell. Regulatory mechanisms for muscle contraction in vertebrates are classified into actin-linked regulation and myosin-linked regulation. Contraction of striated muscle is mainly regulated by the former system while contraction of smooth muscle is mainly regulated by the latter (Szent-Gyorgyi, 1975). Tropomyosin, troponin I, and myosin heavy chain are components of the actin-linked regulation, whereas calmodulin, calponin, myosin light chain kinase, myosin regulatory light chain, and myosin heavy chain are components of myosin-linked regulation. The fact that all of the muscle-related genes described above were expressed in the amphioxus notochord suggests that the muscle-contractile components of the amphioxus notochord are a mixture of vertebrate-type striated and smooth muscle components. This fact may account for the alignment of myofilaments; namely, their alignment is not strictly ordered (Umeda *et al.,* 1999). This may also reflect the uncommon responsibility upon nervous stimulation compared with the myotome muscle (Flood *et al.,* 1969; Flood, 1975).

Actin is a major structural component of the contractile system, both in muscle cells and in nonmuscle cells (Pollard and Cooper, 1986; Sheterline and Sparrow, 1994). Actins are highly conserved proteins found in all eukaryotes, and most organisms have genes that encode several actin isoforms. In vertebrates, for example, two different isoforms are found in nonmuscle cells (cytoplasmic β- and γ-actin) and four isoforms in muscle cells: two specific for striated muscle (α-skeletal and α-cardiac) and two specific for smooth muscle (α-vascular and γ-enteric; Vandekerckhove and Weber, 1984). In vertebrates as well as lower chordates, muscle-type actin is distinguishable from cytoplasmic-type actin by the usage of amino acid residues at 20 diagnostic positions (Vandekerckhove and Weber, 1984; Kovilur *et al.,* 1993; R. Kusakabe *et al.,* 1997; T. Kusakabe *et al.,* 1997).

cDNA clones for both cytoplasmic-type actin and muscle-type actin have been characterized in the amphioxus *B. belcheri* and *B. floridae* (R. Kusakabe *et al.,* 1997, 1999). As shown in Fig. 3B, comparison of the amino acid residues at the diagnostic positions of the amphioxus cytoplasmic-type (BbCA1) actin and muscle-type (BbMA1) actin with those of vertebrate actins suggests that both



types of the amphioxus actins are unique. The amphioxus BbCA1 actin shares only 13 of 20 diagnostic amino acid residues with mammalian β-cytoplasmic actin, whereas the amphioxus BbMA1 actin shares only 11 of the 20 residues with mammalian α-skeletal actin. This contrasts with ascidian actins: *Halocynthia roretzi* cytoplasmic-type actin (HrCA1) (T. Kusakabe *et al.*, 1997) shares 16 of the 20 residues with mammalian cytoplasmic actin, and the *H. roretzi* muscle-type actin (HrMA4) (Araki *et al.*, 1996) shares 18 of the 20 residues with mammalian muscle-type actin. Furthermore, the amphioxus actin (BbNA1) expressed in the notochord is especially unique, because it shares 10 of 20 positions with mammalian β-cytoplasmic actin and 9 of 20 residues with mammalian α-skeletal actin. Therefore, the BbNA1 actin is neither the cytoplasmic-type nor the muscle-type, but rather should be called "amphioxus notochord actin."

In addition, as shown in Fig. 3C, there are 68 nonconserved amino acid sites in amphioxus actins. BbNA1 actin shares 35 of the 68 amino acids with BbCA1 actin and 9 of the 68 amino acids with BbMA1 actin. This suggests that the amphioxus notochord actin more closely resembles the amphioxus cytoplasmic actin. However, it contains 24 amino acid residues different from those of the cytoplasmic and muscle actins. It is likely that one of the actin genes produced by a duplication of the original gene obtained a new expression pattern and function in the notochord.

As shown in Fig. 4C, the 01F12 actin gene is expressed exclusively in the notochord cells. However, PCR analyses suggested that all of the other muscle-related genes isolated from the amphioxus notochord cells are also expressed in the myotome. Do the notochord and myotome use the same set of muscle-related genes or different sets of muscle-related genes? Future studies should identify genes that are expressed in the myotome and determine the temporal and spatial expression patterns of all of the muscle-related genes during embryogenesis, metamorphosis, and juvenile development of this animal.

The Adult Amphioxus Notochord as a Unique Organ

The cephalochordate amphioxus belongs to the phylum Chordata together with urochordates and vertebrates and is the invertebrate group closest to vertebrates (Wada and Satoh, 1994). In vertebrates, a T-box transcription factor gene *Brachyury* is essential for the posterior mesoderm formation and notochord differentiation (see Herrmann and Kispert, 1994; Smith, 1997; Papaioannou and Silver, 1998). In addition, the *Brachyury* gene of urochordate ascidians is expressed in the notochord precursor cells and is a critical factor for the notochord formation (e.g., Yasuo and Satoh, 1993). The expression pattern of the amphioxus *Brachyury* in the presumptive mesoderm, posterior mesoderm, and notochord is almost identical to that of vertebrates (P. Holland *et al.*, 1995; Terazawa and Satoh, 1997). Together with the phylogenetic relationship and the similarity of *Brachyury* expression pattern, it is suggested that the mechanism of notochord development is fundamentally conserved in chordates. Actually, the shape of notochord cells of all three chordate groups is quite similar; e.g., formation of a large vacuole within the cell.

The present EST analysis provided information on genes that are expressed in the amphioxus notochord. Genes that are expressed in the notochord of the ascidian tadpole larva have been characterized as targets of the *Brachyury* gene of *Ciona intestinalis* (Takahashi *et al.*, 1999; Hotta *et al.*, 2000). The results so far obtained, however, suggest that unexpectedly few common genes are expressed in the notochords of both animal groups. A gene for agrin, which is a component of the basal lamina at the neuromuscular junction (Timple and Brown, 1994), and a gene for ATP sulfurylase/APS kinase are two examples. One reason we could not find many genes common to the notochords of the two different chordate groups might be differences in the developmental stage. The ascidian notochord is a larval organ, while the amphioxus notochord examined in the present study is an adult organ. Of course, there have been no reports demonstrating the expression of muscle-related genes in the notochord cells of ascidians (Takahashi *et al.*, 1999; Hotta *et al.*, 2000) and vertebrates.

In the amphioxus notochord cells, myofibrils appear by the time of metamorphosis, with regression of the vacuole in the cytoplasm. It means that the amphioxus notochord changes its cell structure drastically and the role of the notochord changes from embryo to adulthood. The notochord of embryonic chordates has an important function as a source of signals required for many aspects of body plan formation. It influences the formation of adjacent neuroectodermal, mesodermal, and endodermal tissues such as the floor plate, motor neurons, sclerotome, dermamyotome, myotome, heart, pancreas, and hypochord (Bumcrot and McMahon, 1995; Ericson *et al.*, 1997; Seung *et al.*, 1997; Dodd *et al.*, 1998). However, the role of the notochord in the

FIG. 5. Alignments of the amino acid sequences of muscle-related genes of the amphioxus notochord with those of the most similar genes found in BLASTX searches of the GenBank dataset. Dark gray boxes denote residues identical between amphioxus (amphi) and compared genes; light gray boxes indicate residues which are not identical but belong to the same similarity groups (Ala, Ser, Thr; Asp, Glu; Asn, Gln; Arg, Lys; Ile, Leu, Met, Val; and Phe, Tyr, Trp). GenBank accession numbers for the compared sequences are: (A) chick tropomyosin (TPmyosin), P02559; (B) chick troponin I (TnI), P02644; (C) human myosin regulatory light chain (MRLC), NP_006462; (D) *B. floridae* (Bf) CAVP, BAA19429; *B. lanceolatum* (Bl) CAVP, BAA19428; (E) human cysteine and glycine-rich protein 2 (CRP2), NP_001312. Dotted lines and solid lines in (E) indicate the LIM domains and the glycine-rich regions, respectively.

adult amphioxus is completely different. As was shown by the present EST analysis, the amphioxus adult notochord expresses various muscle-related genes and is therefore considered a contractile skeletal organ. It should be noted that the adult amphioxus notochord is a unique organ, which acquires a function different from that of other developing notochord of chordates.

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